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Award Number: W81XWH-09-1-0449

TITLE: Identifying the Mechanism(s) Responsible for the Translational Regulation of the Stress Signaling Kinase MKK4

PRINCIPAL INVESTIGATOR: Carrie W. Rinker-Schaeffer, Ph.D.

CONTRACTING ORGANIZATION: The University of Chicago
Chicago, IL 60637

REPORT DATE: August 2013

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
August 2013	Annual	1 August 2012 - 31 July 2013

4. TITLE AND SUBTITLE
Identifying the Mechanism(s) Responsible for the Translational Regulation of the Stress Signaling Kinase MKK4

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-09-1-0449

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Carrie Rinker-Schaeffer, Ph.D.

E-Mail: crinkers@uchicago.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Chicago
Chicago, IL 60637

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Findings reported herein detail our progress on the development of prostate cancer cell line reagents, which are critical to our proposed studies of micro RNA-mediated regulation of the MKK4 protein. Since our last progress report we have focused on studies in Specific Aim 1, Major Task 2, subtask 1d and 1e. Specifically, building on our previous findings, we now have determined the growth kinetics and metastatic site preference of CWR22Rv1-luc2 cells after intracardiac injection using both IVIS imaging and histology. We have also made significant progress in our efforts to develop bone-seeking cell lines from CWR22Rv1-luc2 cells. Thus far, our effort to develop bone-seeking sublines of the CWR22Rv1 cell line is on track. To be frank, the work in progressing at a faster pace and with cleaner results that we expected. Thus, we are in an excellent position to address the work proposed for YR5 of our EWOF.

15. SUBJECT TERMS
None provided.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	15	19b. TELEPHONE NUMBER *(include area code)*

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INTRODUCTION: Since our last progress report we have focused on studies in Specific Aim 1, Major Task 2, subtask 1d and 1e. Specifically, building on our previous findings, we now have determined the growth kinetics and metastatic site preference of CWR22Rv1-luc2 cells after intracardiac injection using both IVIS imaging and histology. We have also made significant progress in our efforts to develop bone-seeking cell lines from CWR22Rv1-luc2 cells. In this report we present findings from both of these efforts. It should be noted that data presented here is the work of Dr. Erin Howe, the Postdoctoral Fellow who is currently funded by this award. She is an enthusiastic young scientist who is fully committed to improving the lives of men suffering from metastatic prostate cancer.

BODY: Our overall progress on the Research-Specific Tasks specified in our SOW Modifications (06 – 13 – 13) is delineated below. (Please Note: Subtasks in light gray font were completed prior to YR4, however, we included them as they were modified in our modified SOW.)

Aim 1: To test the hypothesis that translational repression of MKK4 is mediated by specific miRNAs (Overall Goal: Preparation of reagents and tools needed to the *in vivo* functional effect of modulating MKK4 protein levels proposed in Aim 2)

➔Major Task 1: Do miRNAs play a role in translational repression of MKK4 in prostate cancer cell lines?

Subtask 1a: Studies to validate the differential expression of candidate miRNAs in prostate cancer cells lines with low MKK4 levels *Cell Lines Used:* PC3, LNCaP, C4-2B, DuPro, Du145, LAPC4, CWR22Rv1, CWR-R1, VCAP

Status: Completed prior to YR 3: Subtask listed as it was modified from the original SOW.

Subtask 1b: Transfection of combinations of precursor (Pre-) miRNAs will be used to decrease the level of MKK4 *Cell Lines Used:* CWR22Rv1, C4-2B

Status: Completed prior to YR 3: Subtask listed as it was modified from the original SOW.

Subtask 1c: antisense (AS-) miRNAs (antagomirs) will be used to increase the level of MKK4 *Cell lines used:* PC 3, C4-2B

Status: Completed prior to YR 3: Subtask listed as it was modified from the original SOW.

Subtask 1e: Validation of MKK4 depletion by treatment with miRNAs using WI38 human fibroblasts a control. *Cell Lines Used:* WI38

Status: Completed prior to YR 3: Subtask listed as it was modified from the original SOW.

Subtask 1f: Lentiviral vectors will be constructed that express: 1) AS-miRNAs to stably deplete endogenous MKK4 protein; and 2) precursor (Pre-) miRNA(s) to stably upregulate endogenous MKK4 protein.

Status: To be done [during EWOF: planned for YR5 months 1-3]

Subtask 1g: Lentiviral vectors will be used to express: 1) shRNAs to stably deplete endogenous MKK4 protein; and 2) MKK4 cDNA to stably express ectopic MKK4 protein.

Status: To be done [during EWOF: planned for YR5 months 1-3]

➔ Major Task 2: Selection and characterization of CWR22Rv1 and C4-2B prostate cancer cell sublines with increased "bone-seeking" ability for use *in vivo* metastasis assays.

Subtask 1a: Construct and characterize CWR22Rv1and C4-2B parental lines with pGL4-luc2 luciferase evaluate photon flux cell lines *in vitro*. *Cell Lines Used:* 2CWR2Rv1, C4-2B

Status: Completed in YR 3

Subtask 1b: Validate the distribution of luciferase-tagged cells after intracardiac injection (state-of-the-art approach for localization of these cell lines to bone). *Cell Lines Used:* CWR22Rv1-luc2, C4-2B-luc2.

Status: Completed in YR 3

Subtask 1c: Conduct dose (cell number injected) response (metastatic yield) curves on luciferase-tagged cells to determine both optimal dose and time-to-endpoint for studies (using IVIS imaging over period of 7 weeks). *Cell Lines Used:* CWR22Rv1-luc2, C4-2B-luc-2.

Status: Completed in YR 3

✓ **Subtask 1d**: Determine the growth kinetics and metastatic site preference of CWR22Rv1-luc2 cells after intracardiac injection using both IVIS imaging and histology. *Cell Lines Used:* CWR22Rv1-luc2.

✓ ***Status:*** Continued during YR 4 [started in YR3]

Summary of Work in Support of Subtask 1d:

In order to determine the growth rate and metastatic potential of CWR22Rv1-luc2 cells, 2.5 x 10^5 cells were injected into the left ventricle of 20 6-8 week old male SCID mice. The larger number of mice in this group enabled quantitation of the spatial location of the metastases that formed. The majority of metastases formed were in the abdominal region of the mice, with 13 out of 18 animals developing metastases in this region (Figure 1, left). Surprisingly, we also saw metastases in the head, which is an undesirable location because of associated morbidity and mortality of the growing lesions. Based upon the published literature, we anticipated that there would be a high incidence of bone metastases in these animals. Thus we were both surprised to find that only 3 animals developed metastases in the long bones of the legs (Figure 1, right). As noted in our previous report, our effort to develop a CWR22Rv1 subline that models clinical prostate cancer bone metastases would be invaluable to both our studies and the greater prostate cancer research community. At that time, we presented the preliminary data in Figure 2. We have included this information in order to provide the reviewer with a full picture of our findings. After intracardiac injection of CWR22Rv1-luc2 cells, mice were followed to the experimental endpoint at which time they were imaged. Long bones showing luciferase positivity were harvested to confirm the presence of cancerous lesions using histology. Femurs of animals that exhibited luciferase signal in the legs were isolated and processed for histologic evaluation by H&E staining.

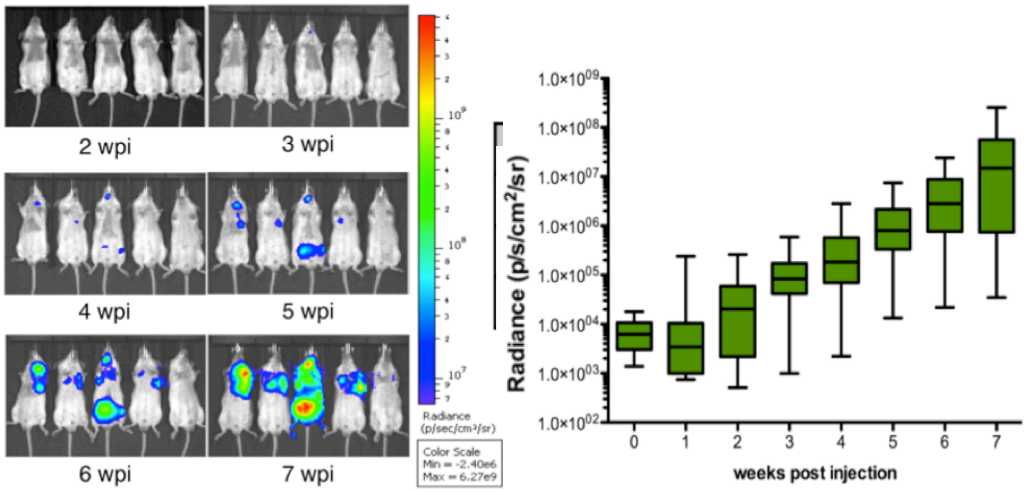


Figure. 1. CWR22Rv1-luc2 growth kinetics and metastatic site preference. ***Left*** 2.5 x 10^5 CWR22Rv1-luc2 cells were injected into the left ventricle of 6-8 week old male SCID mice. Animals were injected ip with luciferin and imaged immediately following injection to ensure injection of the cells into the circulation. Animals were imaged weekly post injection (wpi). The number of animals displaying luciferase signal in the head, abdominal or leg regions is shown at left. ***Right*** Total luciferase radiance was calculated using Living Image software. *Line,* mean, *box*, 25th and 75th percentile, *bars*, range.

Shown in Figure 2A is the IVIS image of an animal that exhibits strong luciferase signal in the leg region. We were elated to find that the CWR22Rv1-luc2 cells were growing in the marrow space of the femur in this animal (Figure 2B). On the top left is an H&E stained section of a normal bone. Observe the linear structure and uniform thickness of the bone itself (light pink). On the top right is a section of bone with CWR22Rv1-luc2 cells growing in the marrow space. Importantly, it appears that the metastases formed by these cells are osteoblastic, which is consistent with the metastatic phenotype observed in clinical disease. The light pink bone is thicker than in the normal control, and is clearly malformed. Note the growth of the bone into the marrow space, with an almost stalagmite appearance. Higher magnification views of the metastases show the abnormal growth of the cells are indicative of metastatic growth (bottom). Further, the cells appear to be forming glandular structures, an observation that warrants further investigation.

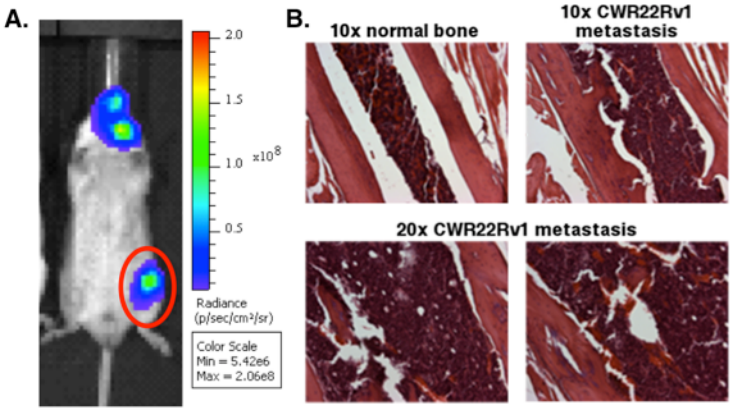


Figure. 2. Validation of CWR22Rv1-luc2 metastatic growth in bone marrow of long bones. A. Close-up of animal undergoing IVIS imaging at 7 weeks post injection. **B.** Femurs of animal dissected out, fixed and decalcified. *Upper left*, H&E of normal bone without CWR22Rv1 growth. *Upper right*, H&E of left femur of animal in A. Malformed bone growth along with metastatic growth in marrow space can be seen. *Lower*, close-up of CWR22Rv1 metastatic growth in bone marrow space. (reproduced from previous annual report).

Additional studies conducted during this EWOF, evaluated the metastatic site preference of the CWR22Rv1-luc2 cells after intracardiac injection. Again, a significant number of animals had positive luciferase signal in the head region (Figure 3 Panels A and B). In order to determine the location of the metastases that correspond to these signals, animals were perfused, the heads were removed, decalcified, formalin-fixed, and paraffin embedded. Tissue sections were H&E stained and evaluated by a pathologist who identified a metastasis in the central region of the brain of animal shown in Panel A (seen as a dark-staining pink region by histology Panels C &D). Other animals, such as the mouse identified in Panel B, had metastases in both the brain (Panel E) and salivary gland (Panel F). Taken together, these data confirmed the presence of brain lesions in a significant number of mice injected with CWR22Rv1-luc2 cells. We anticipate that as we select for sublines with increased bone-seeking activity, the occurrence of metastases in the brain and salivary glands will decrease.

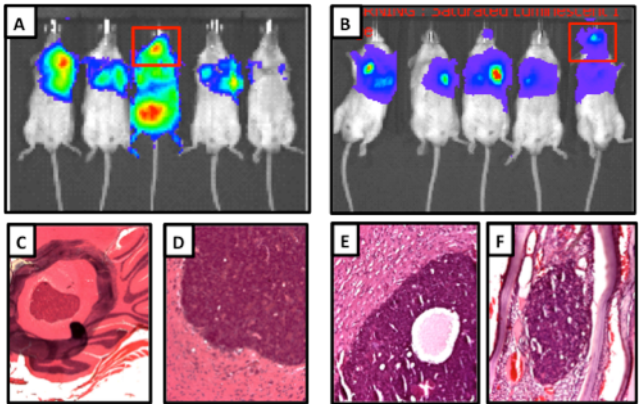


Figure 3. Mice can develop brain and salivary gland metastases after intracardiac injection of CWR22Rv1-luc2 cells. CWR22Rv1-luc2 cells (2.5 x 10^5) were injected into the left ventricle of male SCID mice. **A & B.** Whole body IVIS image of mice at 7 weeks post injection. Mice showing significant signal in brain (examples are indicated by red boxes) were perfused with formalin, the heads removed and decalcified, and finally and formalin-fixed and paraffin-embedded. Sections of the head were evaluated by H&E staining. **B.** 4x image and **C.** 20x image of CWR22Rv1-luc2 cells growing in the brain as identified by a pathologist. The dark pink-staining tumor is circled in black marker**. E.** 20x image of CWR22Rv1-luc2 cells growing in the brain as identified by a pathologist. **F.** 20x image CWR22Rv1-luc2 cells growing in the salivary gland as identified by a pathologist.

Subtask 1e: Serial passaging of CWR22Rv1-luc2 cells through SCID mice via intracardiac injection to select for a subline with increased propensity for bone metastases.

Status: Continued during YR 4 [started in YR3]

Summary of Work in Support of Subtask 1d:

Having confirmed that CWR22Rv1-luc2 cells can form osteoblastic metastases in the long bones, we sought to use them to develop a subline of cells that efficiently colonizes the bone and yields quantifiable numbers of lesions for mechanism-based and therapeutic studies. While development of organotopic sublines of metastatic cells is time-consuming, the methods employed are well-established and reliable. Basically the approach uses repeated rounds of *in vivo* passaging to select for populations of cells that are enriched in their ability to form bone metastases. Cells are injected into animals as before, and the cells that form the desired bone metastases are extracted, cultured and reinjected (Figure 6 A). This methodology has been successfully employed in breast cancer to create lung trophic, bone trophic and brain trophic cell lines from a single parental cell line. To begin our effort, cells were harvested from the metastatic lesion (corresponding to the strong signal) in the left femur (Fig. 7B). The bone marrow from this bone was isolated and grown on tissue culture plates. As the cells grew out of the bone marrow, their derivation from the parental CWR22Rv1-luc2 cell line was confirmed by morphology in culture (Fig. 6C, *left*). Further, the bone marrow derived cells were resistant to hygromyocin (the selection marker on the luciferase plasmid), and they emitted light when given luciferin (Fig. 6C, *right*). Taken together, these results show that the cells we grew from the bone

marrow of a CWR22Rv1-luc2 injected animal were related to the parental cells, and were derived from CWR22Rv1-luc2 cells. We termed these cells CWR22Rv1-luc2-LF cells, after the left femur that they were isolated from.

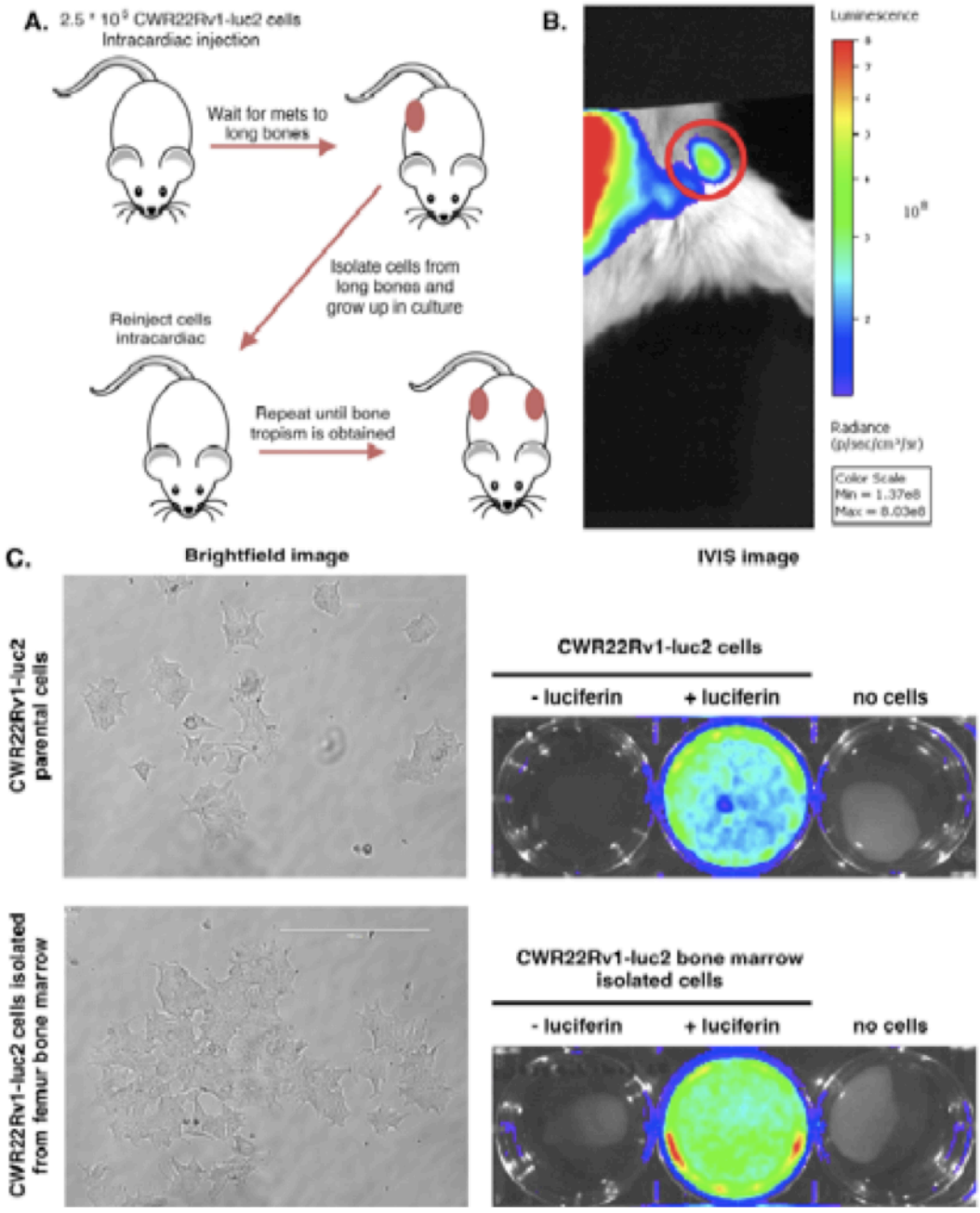


Figure 4. Derivation of bone trophic CWR22Rv1-luc2 cells. A. Conceptual strategy for the derivation of bone trophic CWR22Rv1 cells by in vivo passage. **B.** Animal injected with CWR22Rv1-luc2 cells that formed a metastasis in the femur, highlighted by red circle. 7 weeks post injection the femur was dissected out and cells in the bone marrow were cultured on a plastic dish. **C.** *Upper left*, CWR22Rv1-luc2 parental cells cultured on plastic tissue culture dish, brightfield image showing cell morphology. *Upper right*, CWR22Rv1-luc2 parental cells plated in a 6 well plate and given luciferin as indicated, then imaged on the IVIS system.

We next sought to determine the metastatic capability of these cells, and further to determine if they exhibit increased tropism for the bone microenvironment. We injected 2.5 x 10^5 CWR22Rv1-luc2-LF cells into the left ventricle of 15 6-8 week old male SCID mice as before. The cells exhibit quicker metastasis formation, with metastases forming as early as one-week post injection (Figure 5, *top right*). There is also enhanced formation of bone metastases, with 2 out of 13 animals forming metastases in the leg bones as early as two weeks, as opposed to the 4 weeks seen in the parental cell line. Interestingly, two animals that exhibited metastatic growth at one-week post injection in the abdominal region (Figure 5, *top row, right two animals*) lost the signal the following week. Upon necropsy we found no metastases in this region indicating that the cells had been cleared over time. Close-up images of the legs of the two animals exhibiting signal in the legs clearly show that the signal is originating from the legs (Figure 5, *bottom*). At the experimental endpoint cell lines were derived from these metastases. Hereafter those lines are referred at CWR22Rv1-luc2-BP2 34L and CWR22Rv1-luc2-BP2 34R. These cells were again passaged through mice, that experiment is nearing completion at this time.

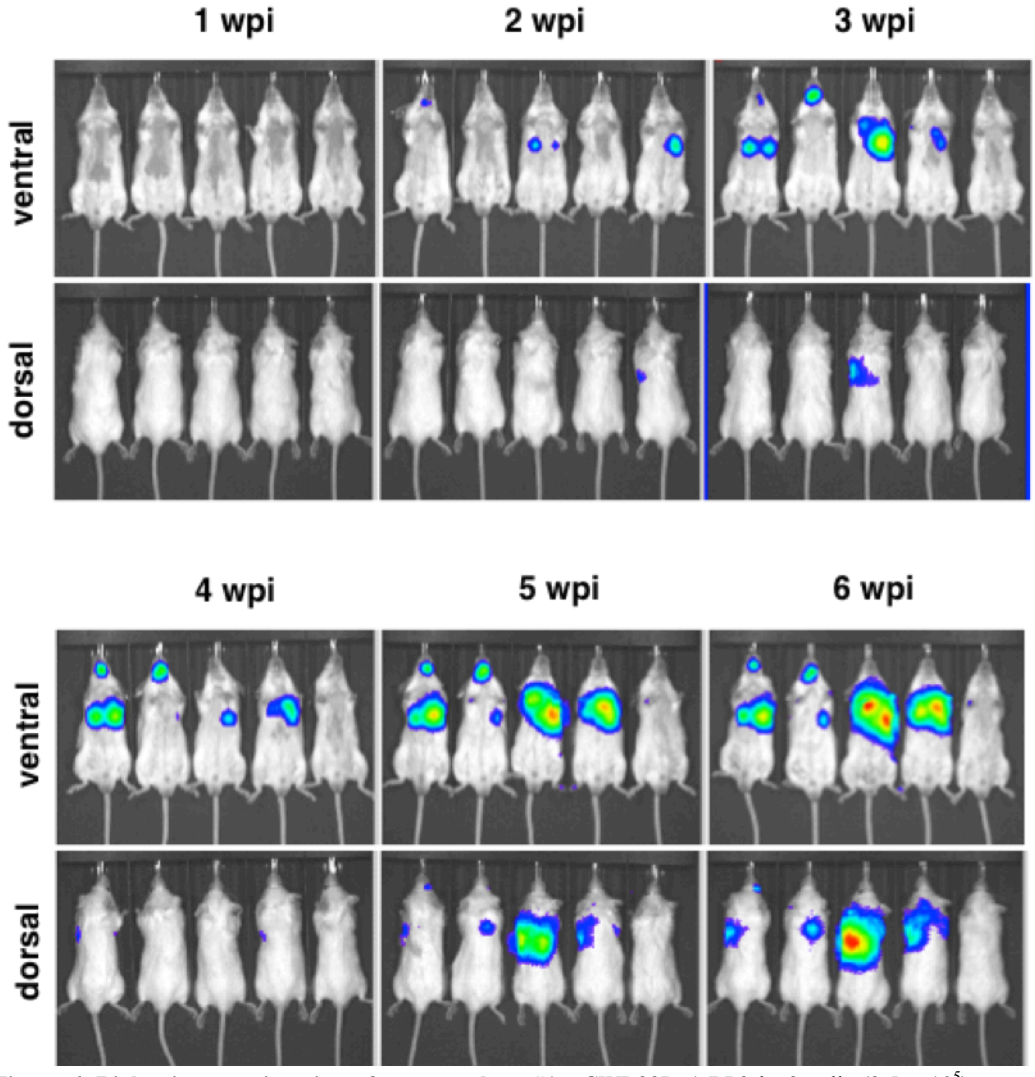


Figure 6. Bioluminescent imaging of mouse cohort #1: CWR22Rv1-BP2-luc2 cells (2.5 x 10^5) were injected into the left ventricle of male SCID mice. Mice were injected with luciferin and IVIS imaged weekly post injection. Rows of images are matched, top two represent ventral and dorsal views of the same animals.

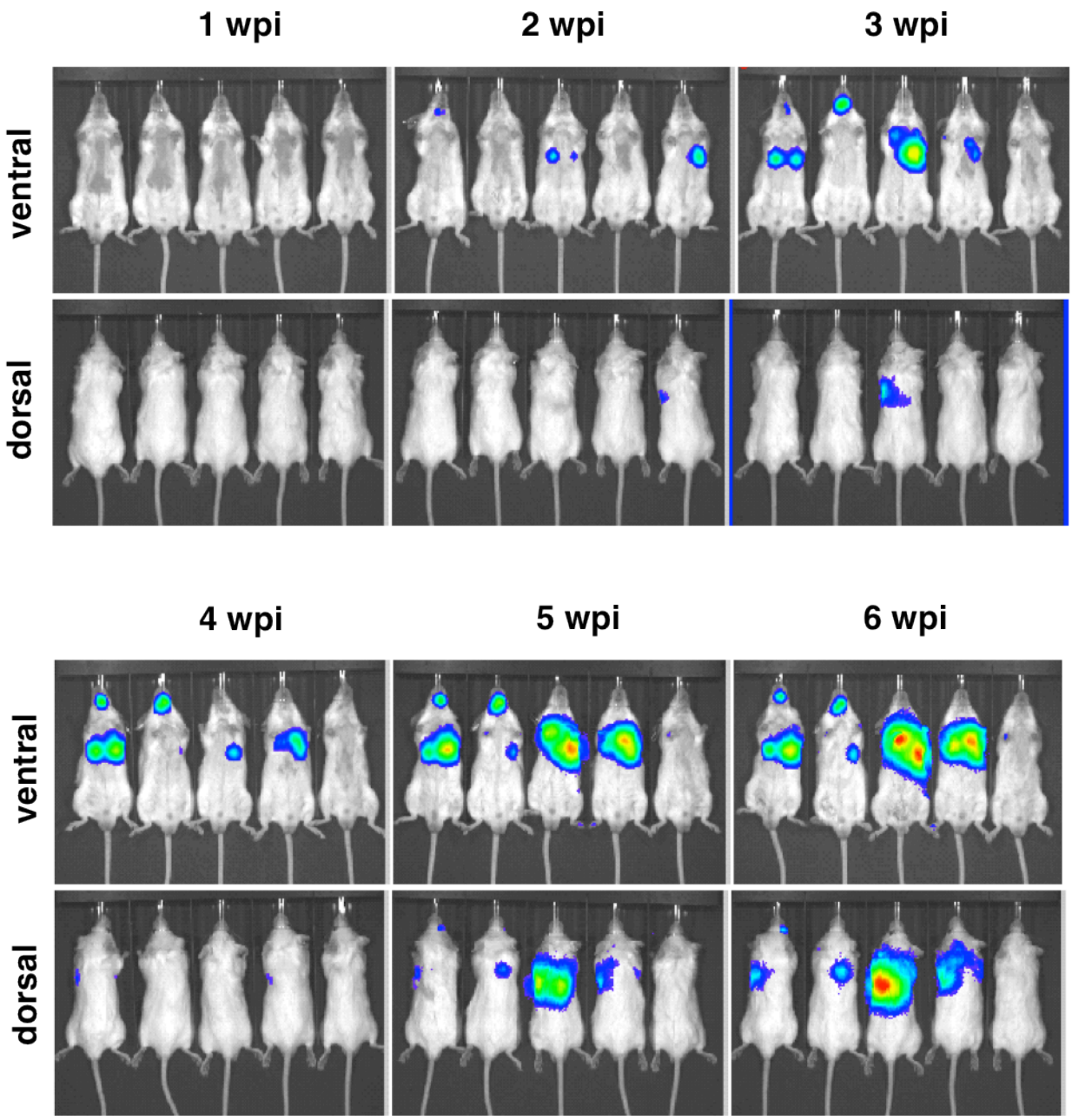


Figure 6. Bioluminescent imaging of mouse cohort #2: CWR22Rv1-BP2-luc2 cells (2.5 x 10^5) were injected into the left ventricle of male SCID mice. Mice were injected with luciferin and IVIS imaged weekly post injection. Rows of images are matched, top two represent ventral and dorsal views of the same animals.

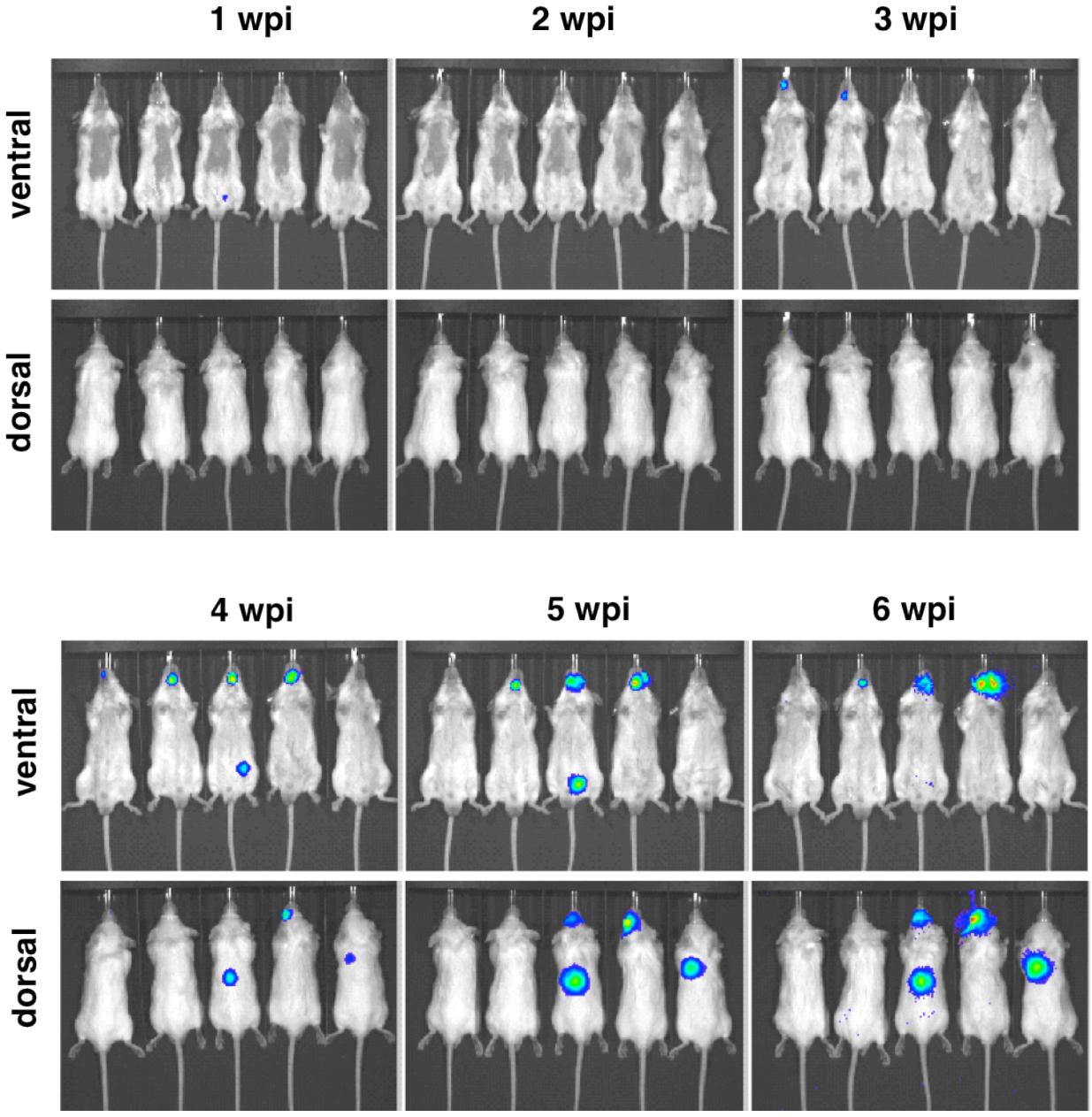
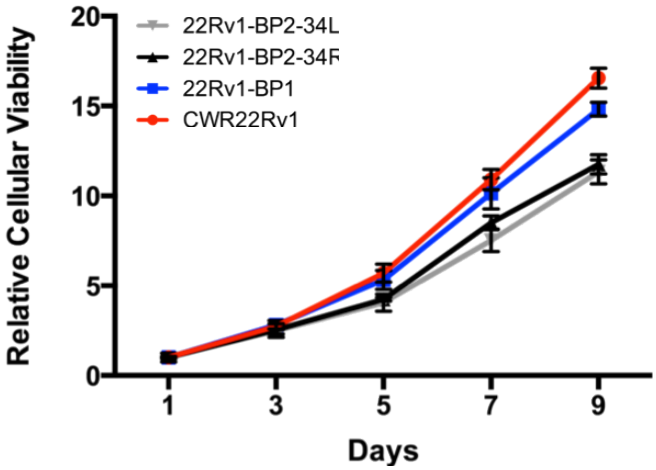


Figure 6. Bioluminescent imaging of mouse cohort #3: CWR22Rv1-BP2-luc2 cells (2.5 x 10^5) were injected into the left ventricle of male SCID mice. Mice were injected with luciferin and IVIS imaged weekly post injection. Rows of images are matched, top two represent ventral and dorsal views of the same animals.

Finally, we assessed the *in vitro* and *in vivo* growth rates of the CWR22Rv1 cell line panel. As shown in Figure 7, the BP-derived CWR22Rv1-luc2-BP1 and CWR22Rv1-luc2-BP2 cells have slopes that are slightly different from that of the CWR22Rv1 parental cell line. This translates into the following doubling times: CWR22Rv1 (60 hrs); CWR22Rv1-BP1 (63 hrs); CWR22Rv1-BP2-34R (67 hrs); and, CWR22Rv1-BP2-34L (65 hrs). These findings are interesting in that generally sublines with increased metastatic ability also show more rapid growth in vitro and *in vivo*. We are anxious to see if this trend continues in our CWR22Rv1-luc2-BP3 cell lines one they are derived and established.

Figure 7. *In vitro* growth rates of CWR22Rv1 cell line panel. CWR22Rv1-luc2, 22Rv1-BP1-luc2, 22Rv1-BP2-34R-luc2 and 22Rv1-BP2-34L-luc2 cells were plated in 96 well plates, 2000 cells per well in quintuplicate. Plates were harvested at timepoints indicated and assayed for cell proliferation via ATP assay (CellTiter-Glo, Promega). *Points*, mean of five replicates, *bars*, standard error of the mean. For each cell line data is normalized to day 1.



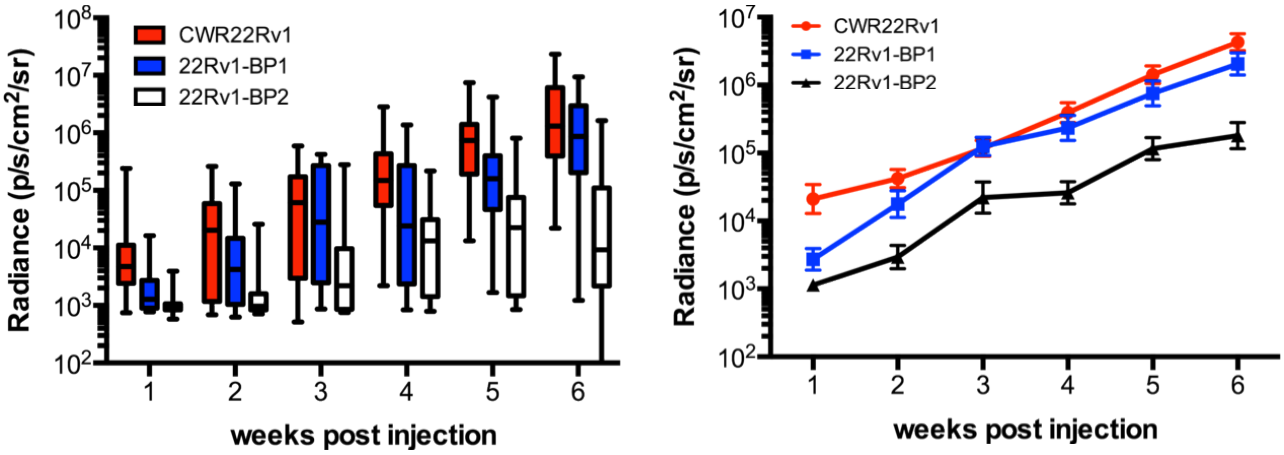


Figure 8. *In vivio* growth rates of CWR22Rv1 cell line panel. CWR22Rv1-luc2, 22Rv1-BP1-luc2, 22Rv1-BP2-34R-luc2 and 22Rv1-BP2-34L-luc2 cells were plated in 96 well plates, 2000 cells per well in quintuplicate. Plates were harvested at timepoints indicated and assayed for cell proliferation via ATP assay (CellTiter-Glo, Promega). *Points*, mean of five replicates, *bars*, standard error of the mean. For each cell line data is normalized to day 1.

Subtask 1f: Characterize CWR22Rv1-luc2-FDIC cells prior to use in Aim 2 (i.e. growth rate, kayotyping, etc.).

Status: To be done [during EWOF: planned for YR5 month 2]

Subtask 1g: Serial passage of C4-2B-luc2 cells *via* direct intratibial injection a subline with increased propensity for growth in bone.

Status: To be done [during EWOF: planned for YR5 month 1-3]

Subtask 1h: Serial passaging of C4-2B-luc2-FD cells *via* intracardiac (IC) injection to select for a sublinewith increased propensity for formation of "spontaneous" bone metastases

Status: To be done [during EWOF: planned for YR5 months 4-7]

Subtask 1i: Characterize C4-2B-luc2-FDIC cells prior to use in Aim 2 (i.e. growth rate, kayotyping, etc.).

Status: To be done [during EWOF: planned for YR5 month 7]

Subtask 1j: Development of a sensitive and specific quantitative reverse transcription PCR (qRTPCR) assay to quantitate the number of prostate cancer cells within the bone. *Cell Lines Used:* (genomic DNA: CWR22Rv1, ID-8).

Status: Completed in Year 3

Subtask 1k: Confirmation that qRT-PCR assay/primers is efficient and linear over a range of template concentrations. *Cell Lines Used:* (genomic DNA: CWR22Rv1, ID-8).

Status: Completed in Year 3

Subtask 1l: Determination of the limit of detection for human prostate cancer cells in the mouse femur. *Cell Line Used:* CWR22Rv1

Status: Completed in Year 3

Subtask 1m: Quantitating skeletal distribution of CWR22Rv1-luc2 cells at discrete time points using qRT-PCR.

Status: To be done [during EWOF: planned for YR5 months 1-6]

Aim 2: To test the hypothesis that modulation of MKK4 levels by miRNAs affects *in vivo* measures of malignant potential.

➔**Major Task 1: Modulation of MKK4 protein levels in prostate cancer cell lines with increased propensity for bone metastasis (Derived in Aim 1)**

Subtask 1a: Use vectors described in Aim1 Major Task 1, subtasks 1f and 1g to modulate MKK4 protein levels in CWR22Rv1-luc2-FDIC cell lines

Status: To be done [during EWOF: planned for YR5 months 3-6]

Subtask 1b: Characterize cell lines generated in Aim 2, Major Task 1, Subtask 1a: 1) confirm MKK4 depletion, and 2) determine *in vitro* growth rates.

Status: To be done [during EWOF: planned for YR5 months 3-6]

Subtask 1c: Validate the distribution of cell lines characterized in Aim 2, Major Task 1, Subtask 1b using IVIS imaging.

Status: To be done [during EWOF: planned for YR5 months 7-12]

Subtask 1d: Determine the growth kinetics and metastatic site preference of cell lines characterized in Aim 2, Major Task1, Subtask 1b using IVIS imaging.

Status: To be done [during EWOF: planned for YR5 months 7-12]

Subtask 1e: Evaluate tissues at endpoint of study in Aim 2, Major Task1, Subtask 1d. Use appropriate methods to quantify metastasis formation (i.e. q-RTPCR, histology, histomorphometry, etc.).

Status: To be done [during EWOF: planned for YR5 months 7-12]

Subtask 1f: Use vectors described in Aim1 Major Task 1, subtasks 1f and 1g to modulate MKK4 protein levels in C4-2B-luc2-FDIC cell lines.

Status: To be done [during EWOF: planned for YR5 months 8-10]

Subtask 1g: Characterize cell lines generated in Aim 2, Major Task 1, Subtask 1f: 1) confirm MKK4 depletion, and 2) determine *in vitro* growth rates.

Status: To be done [during EWOF: planned for YR5 months 8-10]

Subtask 1h: Validate the distribution of cell lines characterized in Aim 2, Major Task 1, Subtask 1g using IVIS imaging.

Status: To be done [during EWOF: planned for YR5 months 9-12]

Subtask 1i: Determine the growth kinetics and metastatic site preference of cell lines characterized in Aim 2, Major Task1, Subtask 1g using IVIS imaging.

Status: To be done [during EWOF: planned for YR5 months 9-12]

Subtask 1j: Evaluate tissues at endpoint of study in Aim 2, Major Task1, Subtask 1i. Use appropriate methods to quantify metastasis formation (i.e. q-RTPCR, histology, histomorphometry, etc.)

Status: To be done [during EWOF: planned for YR5 months 7-12]

KEY RESEARCH ACCOMPLISHMENTS:

- Established the growth kinetics and metastatic site preference for CWR22Rv1-luc2 cells after intracardiac injection of $2.5x10^5$ cells.
- Confirmed that intracardiac injection of $2.5x10^5$ cells results in formation of brain metastases in a significant number of mice within a cohort.
- Completed two rounds of passaging of the original CWR22Rv1-luc2 cells to select for sublines with increased propensity for bone metastasis formation. This ultimately resulted in the development of the CWR22Rv1-luc2-BP1 and CWR22Rv1-luc2-BP2 sublines. The selection for and establishment of the CWR22Rv1-luc2-BP3 cell line in nearly complete.
- Determined the *in vitro* and in vivo growth rates of a panel of CWR22Rv1-luc2 panel of cell lines.

REPORTABLE OUTCOMES:

None

CONCLUSIONS AND FUTURE DIRECTIONS: CONTINUING EFFORTS: A continuing challenge to researchers in the prostate cancer metastasis community is having models that closely parallel clinical disease. Thus far, our effort to develop bone-seeking sublines of the CWR22Rv1 cell line is on track. To be frank, the work in progressing at a faster pace and with cleaner results that we expected. This is great news for our work and for the prostate cancer research community. We are poised to complete the work proposed for YR EWOF. We appreciate the opportunity to see this work to fruition and make a valuable contribution to the area of prostate cancer metastasis research.

REFERENCES:

None

APPENDICIES:

None